α-AMYLASE ASSAY AND USES THEREOF

Related Applications

This application is a continuation-in-part of United States application serial number 10/230,969, filed August 29, 2002, now pending.

Field of the Invention

This invention relates to methods and kits for measuring α-amylase activity in grain products such as flour.

Background of the Invention

The use of flour produced through the processing of cereal grains such as wheat, rye, and oats is an important feature in nutrition and food production around the world. Grains are grown, harvested, and milled into flours, which are used to make breads, bakery products, pastas, all of which are staples in the diet of many individuals world wide. Grains and grain products are also utilized for brewing and fermentation.

Wheat flour is an important ingredient in home baking and is the foundation for almost every commercially baked product and pasta. Of the grains available for the production of flour, wheat is unique in that it is the only cereal grain with sufficient gluten content to make a loaf of bread without being mixed with another grain. Wheat is grown all over the world and is the most widely distributed cereal grain. In general, a reference to "flour" is a reference to wheat flour. Flour is used extensively in the food industry and a key requirement in that industry is the uniform high quality and performance of flour and grains in food and beverage production (For review see: *Plant Foods for Human Nutrition*, Vol 55:1-86, 2000).

Cereal grains store energy as starch, and to perform well in baking and food production, it is important to optimize the level of starch in flour. A key factor in the breakdown of starch in flours is the presence of α-amylase activity in cereal grain flours. α-amylase is an endoenzyme that is present in cereal grains and breaks the α-1,4, glucosidic bonds that are present in starch. The enzyme works in an almost

random manner and the effect of its enzymatic activity is the breakdown in the size of the starch molecules and the conversion of starch to sugars and dextrins.

To help ensure efficient food production methods, it is important to be able to accurately assess the level of α-amylase activity in batches of flour. The presence of excess α-amylase activity flour results in a reduction in the value of the flour for baking. For example, excess starch breakdown in flour can result in sticky or doughy bread that can't be cut in automated loaf-slicing machinery and is therefore unsuitable for commercial production. Insufficient α-amylase activity in flour can also reduce the value of a flour for baking and food production. Insufficient α-amylase activity in flour can result flour that lacks the necessary levels of sugars for proper fermentation and yeast activity in baking. Flours with insufficient α-amylase are frequently supplemented with amylase concentrates. Because of the financial importance of flour quality in the baking and food production industries, it is important to have reliable, reproducible, and easy-to-use methods to determine the amount of α-amylase activity in flours.

Current methods to determine the level of α-amylase in flour include techniques such as the Hagberg-Perten Falling Number test. This is a viscosity-based method in which a flour suspension is heated to gelatinize the starch. The viscosity of the mixture is determined by putting the suspension into a long narrow tube of defined dimensions and measuring the rate at which various calibrated small stirrers or a rod falls though the suspension in the tube. Although the Falling Number test is currently accepted as the industry standard, it does not measure the actual α-amylase enzyme activity level directly, and it is the activity of the enzyme that affects baked good texture and value.

Alternative methods that directly measure α-amylase enzyme activity have been developed, but are not used to fluorometrically test flour, amylase concentrate, or stock samples, which limits their usefulness. The availability of a fluorometric method to directly determine α-amylase enzyme activity in flour, amylase concentrates, or stock samples would provide a more accurate prediction of a flour's, concentrate's, or stock's performance, and therefore its value in the baking industry and in other food and beverage production industries.

Summary of the Invention

The invention is based, in part, on our surprising discovery that the level of α-amylase enzyme activity in a flour sample and/or amylase concentrate sample can be determined by contacting a sample from a grain flour or other grain or plant product, or an amylase concentrate sample, with a detectably labeled starch substrate and determining the amount of hydrolysis of the substrate as a measure of the α-amylase enzyme activity in the sample.

According to one aspect of the invention, methods for measuring α-amylase activity in a sample are provided. The methods include forming a reaction mixture by contacting a sample with a detectably labeled starch substrate for a time sufficient for α-amylase in the sample to hydrolyze the starch substrate, thereby releasing soluble detectably labeled starch fragments, separating the soluble detectably labeled starch fragments from the reaction mixture, and determining the level of hydrolysis of the detectably labeled starch substrate as a measurement of α-amylase activity in the flour or stock sample. In some embodiments, the sample is a flour sample. In certain embodiments, the sample is a stock sample. In some embodiments, the sample is an amylase concentrate sample.

In some embodiments, determining the level of hydrolysis of the detectably labeled starch substrate includes quantifying the detectably labeled starch substrate. In other embodiments, determining the level of hydrolysis of the detectably labeled starch substrate includes quantifying the soluble detectably labeled starch substrate fragments. In some embodiments, the method also includes calculating the α-amylase activity in the sample by correlating the quantity of detectably labeled starch to an α-amylase standard. In some embodiments, the method also includes calculating the α-amylase activity in the sample by correlating the quantity of soluble detectably labeled starch fragments to an α-amylase standard. In certain embodiments, the detectably labeled starch substrate is a potato starch. In certain embodiments, the detectably labeled starch substrate includes D-glucose residues and is labeled on about one of every 300-1300 D-glucose residues of the starch substrate. In some embodiments, the starch substrate is detectably labeled with a label compound selected from the group consisting of fluorescent, enzyme, radioactive, metallic, biotin, chemiluminescent, and bioluminescent molecules. In some embodiments, the

label is a fluorophore. In certain embodiments, the fluorophore is selected from the group consisting of 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS), fluorescein isothiocyanate (FITC), and Marina Blue.

In some embodiments, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes filtering the reaction mixture to remove from the mixture detectably labeled starch substrate. In some embodiments, the step of filtering includes the addition of a filtration aid selected from the group consisting of resin, glass beads, beads, and celite. In some embodiments, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes centrifuging the reaction mixture to remove from the mixture detectably labeled starch substrate. In some embodiments, the method also includes measuring an aliquot of the supernatant of the centrifuged reaction mixture. In certain embodiments, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes obtaining an aliquot of the reaction mixture and centrifuging the aliquot of the reaction mixture to remove from the aliquot detectably labeled starch substrate. In some embodiments, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes contacting the fragments with an agent that binds to the detectably labeled starch fragments. In certain embodiments, the agent is a lectin. In other embodiments, the agent is an antibody.

In some embodiments, the sample is an aqueous slurry. In some embodiments, the sample is contacted with the detectably labeled starch substrate for a reaction time of at least about 1 sec, 5 sec, 10 sec, 15 sec, 20 sec, 25 sec, 30 sec, 35 sec, 40 sec, 45 sec, 50 sec, 55 sec, 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 11 min, 12 min, 13 min, 14 min, 15 min, 16 min, 17 min, 18 min, 19 min, 20 min, 21 min, 22 min, 23 min, 24 min, 25 min, 26 min, 27 min, 28 min, 29 min, 30 min, 31 min, 32 min, 33 min, 34 min, 35 min, 36 min, 37 min, 38 min, 39 min, 40 min, 41 min, 42 min, 43 min, 44 min, 45 min, 46 min, 47 min, 48 min, 49 min, 50 min, 51 min, 52 min, 53 min, 54 min, 55 min, 56 min, 57 min, 58 min, 59 min, or 60 min. Preferably, the sample is contacted with the detectably labeled starch substrate for a reaction time at least about 1 minute, at least about 5 minutes, at least about 10 minutes, or at least about 15 minutes.

According to another aspect of the invention, methods for measuring α-amylase activity in a sample are provided. The methods include forming a reaction mixture by contacting a sample with a detectably labeled starch substrate attached to a surface, for a time sufficient for α-amylase in the sample to hydrolyze the starch substrate, thereby releasing soluble detectably labeled starch fragments, separating the soluble detectably labeled starch fragments from the reaction mixture, and determining the level of hydrolysis of the detectably labeled starch substrate as a measurement of α-amylase activity in the sample. In some embodiments, the sample is a flour sample. In other embodiments, the sample is a stock sample. In some embodiments the sample is an amylase concentrate sample.

In some embodiments, the surface is selected from the group consisting of a tube, a centrifuge tube, a cuvette, a dipstick, a multiwell plate, a slide, a coverslip, a card, a bead, and a plate. In some embodiments, determining the level of hydrolysis of the detectably labeled starch substrate includes quantifying the soluble detectably labeled starch substrate fragments. In some embodiments, the method also includes calculating the α-amylase activity in the sample by correlating the quantity of soluble detectably labeled starch fragments to an α-amylase standard. In some embodiments, the α-amylase standard is an α-amylase standard curve.

In some embodiments, determining the level of hydrolysis of the detectably labeled starch substrate includes quantifying the detectably labeled starch substrate after separating the soluble detectably labeled starch fragments from the reaction mixture. In some embodiments, the method also includes releasing the detectably labeled starch substrate from the surface after separating the soluble detectably labeled starch fragments from the reaction mixture. In certain embodiments, determining the level of hydrolysis of the detectably labeled starch substrate includes quantifying the detectably labeled starch substrate after releasing the detectably labeled starch substrate from the surface. In some embodiments, the method also includes calculating the α-amylase activity in the sample by correlating the quantity of detectably labeled starch to an α-amylase standard. In some embodiments, the detectably labeled starch substrate is a potato starch.

In some embodiments, the detectably labeled starch substrate includes D-glucose residues and is labeled on about one of every 300-1300 D-glucose residues of

the starch substrate. In some embodiments, the starch substrate is detectably labeled with a label compound selected from the group consisting of fluorescent, enzyme, radioactive, metallic, biotin, chemiluminescent, and bioluminescent molecules. In some embodiments, the label is a fluorophore. In some embodiments, the fluorophore is selected from the group consisting of 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS), FITC, and Marina Blue.

In some embodiments, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes filtering the reaction mixture to remove from the mixture detectably labeled starch substrate. In certain embodiments, the step of filtering includes the addition of a filtration aid selected from the group consisting of resin, glass beads, beads, and celite. In some embodiments, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes centrifuging the reaction mixture to remove from the mixture detectably labeled starch substrate. In some embodiments, the method also includes measuring an aliquot of the supernatant of the centrifuged reaction mixture. In some embodiments, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes obtaining an aliquot of the reaction mixture and centrifuging the aliquot of the reaction mixture to remove from the aliquot detectably labeled starch substrate. In certain embodiments, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes contacting the fragments with an agent that binds to the detectably labeled starch fragments. In some embodiments, the agent is a lectin. In some embodiments, the agent is an antibody.

In some embodiments, the sample is an aqueous slurry. In some embodiments, the sample is contacted with the detectably labeled starch substrate for a reaction time of at least about 1 sec, 5 sec, 10 sec, 15 sec, 20 sec, 25 sec, 30 sec, 35 sec, 40 sec, 45 sec, 50 sec, 55 sec, 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 11 min, 12 min, 13 min, 14 min, 15 min, 16 min, 17 min, 18 min, 19 min, 20 min, 21 min, 22 min, 23 min, 24 min, 25 min, 26 min, 27 min, 28 min, 29 min, 30 min, 31 min, 32 min, 33 min, 34 min, 35 min, 36 min, 37 min, 38 min, 39 min, 40 min, 41 min, 42 min, 43 min, 44 min, 45 min, 46 min, 47 min, 48 min, 49 min, 50 min, 51 min, 52 min, 53 min, 54 min, 55 min, 56 min, 57 min, 58 min, 59

min, or 60 min. Preferably, the sample is contacted with the detectably labeled starch substrate for a reaction time of at least about 1 minute, at least about 5 minutes, at least about 10 minutes, or at least about 15 minutes.

According to yet another aspect of the invention, kits for measuring α-amylase activity in a sample are provided. The kits include a first container containing a detectably labeled starch substrate, a second container containing an α-amylase standard, instructions for measuring the α-amylase activity in a sample.

According to another aspect of the invention, kits for measuring α-amylase in a sample are provided. The kits include a first container containing a detectably labeled starch substrate, calibration standards, and a conversion table or curve for converting fluorometer readings to amylase units.

In some embodiments of the foregoing kits, the sample is a flour sample. In other embodiments of the foregoing kits, the sample is a stock sample. In some embodiments of the foregoing kits the sample is an amylase concentrate sample. In some embodiments of the foregoing kits, the starch substrate is potato starch. In some embodiments of the foregoing kits, the detectably labeled starch substrate includes D-glucose residues and is labeled on about one of every 300-1300 D-glucose residues of the starch substrate. In some embodiments of the foregoing kits, the detectable label is a label compound selected from the group consisting of fluorescent, enzyme, radioactive, metallic, biotin, chemiluminescent, and bioluminescent molecules. In certain embodiments of the foregoing kits, the starch substrate is detectably labeled with a label compound selected from the group consisting of fluorescent, enzyme, radioactive, metallic, biotin, chemiluminescent, and bioluminescent molecules. In some embodiments of the foregoing kits, the label is a fluorophore. In certain embodiments of the foregoing kits, the fluorophore is selected from the group consisting of 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS), FITC, and Marina Blue. In some embodiments of the foregoing kits, the instructions for measuring the α-amylase activity in a sample recite a method comprising forming a reaction mixture by contacting a sample with a detectably labeled starch substrate for a time sufficient for α-amylase in the sample to hydrolyze the starch substrate, thereby releasing soluble detectably labeled starch fragments, separating the soluble detectably labeled starch fragments from the reaction mixture, and quantifying the

soluble detectably labeled starch as a measurement of α-amylase activity in the sample. In some embodiments of the foregoing kits, the instructions further recite calculating the α-amylase activity in the sample by correlating the quantity of soluble detectably labeled starch fragments to an α-amylase standard. In some embodiments, the α-amylase standard is an α-amylase standard curve.

In some embodiments of the foregoing kits, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes filtering the reaction mixture to remove from the mixture detectably labeled starch substrate. In some embodiments of the foregoing kits, the step of filtering includes the addition of a filtration aid selected from the group consisting of resin, glass beads, beads, and celite. In some embodiments of the foregoing kits, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes centrifuging the reaction mixture to remove from the mixture detectably labeled starch substrate. In some embodiments of the foregoing kits, the step also includes measuring an aliquot of the supernatant of the centrifuged reaction mixture. In certain embodiments of the foregoing kits, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes obtaining an aliquot of the reaction mixture and centrifuging the aliquot of the reaction mixture to remove from the aliquot detectably labeled starch substrate. In some embodiments of the foregoing kits, the step of separating the soluble detectably labeled starch fragments from the reaction mixture includes contacting the fragments with an agent that binds to the detectably labeled starch fragments. In some embodiments of the foregoing kits, the agent is a lectin. In other embodiments of the foregoing kits, the agent is an antibody.

In some embodiments of the foregoing kits, the instructions further recite that the sample is an aqueous slurry. In some embodiments of the foregoing kits, the instructions further recite that the sample is contacted with the detectably labeled starch substrate for a reaction time of at least about 1 sec, 5 sec, 10 sec, 15 sec, 20 sec, 25 sec, 30 sec, 35 sec, 40 sec, 45 sec, 50 sec, 55 sec, 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 11 min, 12 min, 13 min, 14 min, 15 min, 16 min, 17 min, 18 min, 19 min, 20 min, 21 min, 22 min, 23 min, 24 min, 25 min, 26 min, 27 min, 28 min, 29 min, 30 min, 31 min, 32 min, 33 min, 34 min, 35 min, 36 min, 37 min, 38 min, 39 min, 40 min, 41 min, 42 min, 43 min, 44 min, 45 min, 46

min, 47 min, 48 min, 49 min, 50 min, 51 min, 52 min, 53 min, 54 min, 55 min, 56 min, 57 min, 58 min, 59 min, or 60 min. Preferably, in some embodiments of the foregoing kits, the instructions recite that the sample is contacted with the detectably labeled starch substrate for a reaction time at least about 1 minute, at least about 5 minutes, at least about 10 minutes, or at least about 15 minutes.

According to yet another aspect of the invention, methods of determining amylase in a sample are provided. The methods include placing about 6ml incubation buffer in a substrate tube, warming the substrate tube to 45°C, adding about 200 mg of the sample to the warmed substrate tube, incubating the sample mixture in the substrate tube 10 min at 45°C, adding about 4 ml stop buffer to the sample mixture in the substrate tube, filtering the stopped sample mixture into a container, determining the fluorescence in the filtrate, and optionally converting the fluorescence value into a Falling Number Equivalent value. In some embodiments, the container is a cuvette. In certain embodiments, the fluorescence is determined in a fluorometer. In some embodiments, the amylase comprises one or more amylases selected from the group consisting of cereal amylase, bacterial amylase, and fungal amylase. In some embodiments, the sample selected from the group consisting of a flour sample, a stock sample, and an amylase concentrate sample. In certain embodiments, the filtering is filtering through a microfiber filter.

According to another aspect of the invention, methods of determining amylase in a sample are provided. The methods include placing an about 3g sample into a first container, adding a sufficient amount of fungal incubation buffer to have the total weight of sample plus buffer equal of about 30g, mixing the solution, extracting the solution for 5 minutes at 45°C, adding the about 8ml of the extract to a substrate tube, incubating extract in substrate tube 10 minutes at 45°C, adding about 2ml stop buffer the tube, mixing the contents of the tube, filtering the mixture into a second container, determining the fluorescence in the filtrate, and optionally converting the fluorescence value into an Enzyme Units Equivalent value. In some embodiments, the first container is a tube. In certain embodiments, the second container is a cuvette. In some embodiments, the fluorescence is determined in a fluorometer. In some embodiments, the sample comprises one or more amylases selected from the group consisting of cereal amylase, bacterial amylase, and fungal amylase. In some

embodiments, the sample is selected from the group consisting of a flour sample, a stock sample, and an amylase concentrate sample. In some embodiments, the flour sample is a wheat flour sample. In some embodiments, the filtering is filtering through a microfiber filter.

According to yet another aspect of the invention, methods of determining amylase in a sample are provided. The methods include placing about 200mg of the sample into a container, adding about 20ml fungal incubation buffer to the sample, mixing the sample solution, diluting about 2ml of the solution with 10ml incubation buffer in a container, optionally further diluting the diluted solution to obtain a concentration within range of about 0.1-1.0 SKB unit/ml, placing about 8ml of the diluted sample into a container, incubating the about 8ml diluted sample 10 minutes at 45°C, adding about 2ml stop buffer to the 8ml diluted sample, filtering the mixture through a filter into a detection container, determining the fluorescence in the filtrate, and optionally converting the fluorescence value into an Enzyme Units Equivalent value and multiplying by the dilution factor as a measure of the original amylase concentration. In certain embodiments, the container is a tube. In some embodiments, the detection container is a cuvette. In some embodiments, the fluorescence is determined in a fluorometer. In certain embodiments, the sample comprises one or more amylases selected from the group consisting of cereal amylase, bacterial amylase and fungal amylase. In some embodiments, the sample is selected from the group consisting of a flour sample, a stock sample, and an amylase concentrate sample. In some embodiments, the flour sample is a wheat flour sample. In some embodiments, the filtering is filtering through a microfiber filter.

According to another aspect of the invention, kits are provided. The kits include a container containing a detectably labeled starch substrate, a standard, and/or a standard curve and/or a conversion table for converting fluorometer readings to amylase units, and instructions for using any of the aforementioned methods to determine the amount of α-amylase activity in a sample. In some embodiments, the kits may also include buffers, tubes, calibration solutions, filters, and/or control samples. In some embodiments, the sample is selected from the group consisting of a flour sample, a stock sample, and an amylase concentrate sample.

These and other aspects of the invention, as well as various embodiments thereof, will become more apparent in reference to the drawings and detailed description of the invention.

Brief Descriptions of the Drawings

Fig. 1 is a graph of results of amylase assay of flour samples at various concentrations versus the Falling Number of the samples.

Fig. 2 is a graph of the results of a determination of Doh Tone™ concentration in wheat flours with the amylase assay.

Fig. 3 shows a graph of the results of a determination of pure Doh Tone™ II (Code 416) with the amylase assay versus concentration.

Fig. 4 is a graph of the results of determination of Doh Tone™ (Code 416) versus concentration to fit a quadratic curve.

Fig. 5 is a graph of the results of determination of Doh Tone™ (Code 416) versus concentration to fit to a linear relation.

Fig. 6 is a graph illustrating the effect of using supernatants of a flour extract instead of flour suspensions as with the amylase assay.

Fig. 7 is a graph illustrating the effect of a stability test performed on AMYLease™ substrate at elevated temperature.

Fig. 8 is a table (Fig. 8A)and graph (Fig. 8B) illustrating the results of determination of fungal amylase from Sigma-Aldrich (St. Louis, MO).

Fig. 9 shows graphs of (Fig.9A) results of the amylase assay on Sigma fungal amylase at high range of amylase units and (Fig. 9B) results of the amylase assay on Sigma fungal amylase at low range of amylase units.

Fig. 10 is a graph illustrating the effects of cereal amylase on determination of fungal amylase with the amylase assay.

Detailed Description of the Invention

For the commercial and home use of flour for baking and food production, it is important to maintain an appropriate level of α-amylase activity in the flour. A level of activity that is too high may result in a product that is sticky and/or doughy and unmarketable; but flour with insufficient α-amylase activity may not contain enough sugar for proper yeast function, resulting in dry, crumbly bread. To augment the level of endogenous α-amylase activity in flour, exogenous (e.g. substitute) α-amylase may be added to flour in the form of fungal α-amylase or other α-amylase. Therefore, the ability to determine the level of activity of both endogenous (natural) and fungal α-amylase, or other α-amylase, in a flour sample would benefit the food production process and promote more efficient use of flour in food production.

In addition to the use of grains and other plant products in baking, grains such as barley, oats, wheat, as well as plant components such as corn, hops, and rice are used for brewing, both in industry and for home brewing. The components used in brewing may be unmalted or may be malted, which means partially germinated resulting in an increase in the levels of enzymes including α-amylase. For successful brewing, adequate levels of α-amylase enzyme activity are necessary to ensure the appropriate levels of sugars for fermentation.

As used herein, the term "flour" means milled or ground cereal grain. The term "flour" may also mean Sago or tuber products that have been ground or mashed. In some embodiments, flour may also contain components in addition to the milled or mashed cereal or plant matter. An example of an additional component, although not intended to be limiting, is a leavening agent. Cereal grains include: wheat, oat, rye, and barley, In preferred embodiments of the invention, the cereal grain is wheat. Tuber products include tapioca flour, cassava flour, and custard powder. The term "flour" also includes ground corn flour, maize-meal, rice flour, whole-meal flour, self-rising flour, tapioca flour, cassava flour, ground rice, and custard powder.

As used herein, the term "stock" means grains and plant components that are crushed or broken. For example, barley used in beer production is a grain that has been coarsely ground or crushed to yield a consistency appropriate for producing a mash for fermentation. As used herein, the term "stock" includes any of the aforementioned types of plants and grains in crushed or coarsely ground forms. It will be understood that the methods of the invention may be used to determine α-amylase activity levels in flours, and also in stock, which includes the aforementioned types of grains, tubers, and other plant products that have been crushed.

As used herein, the term "amylase concentrate" means a sample that includes amylase. The amylase may be fungal, bacterial, and/or cereal amylase. It will be understood that the methods of the invention may be used to determine the amount or level of α-amylase activity in a sample that contains a single amylase and/or a sample that contains more than one type of amylase, for example, the sample may include fungal α-amylase or may include cereal and fungal α-amylase.

The invention involves in some aspects, methods for measuring α-amylase activity in flour, grain or tuber products, stock, and amylase concentrate samples. As used herein, the term "α-amylase" means endogenous α-amylase, exogenous α-amylase (e.g. α-amylase concentrate), or α-amylase that has been added to flour or stock. As used herein, the term "α-amylase" means a protein having α-amylase activity, preferably plant-derived α-amylase and/or microbial α-amylase. Plant-derived α-amylase includes, but is not limited to, cereal α-amylase and wheat α-amylase. Microbial α-amylase includes, but is not limited to, bacterial α-amylase, and fungal α-amylase. As used herein, the term "α-amylase activity" means the enzymatic action of the α-amylase. The enzymatic action of the α-amylase includes the hydrolysis (breakage) of the α-1,4, glucosidic bonds present in starch, which reduces the size of the starch molecules and converts the starch into sugar.

The invention involves in some aspects, contacting a sample with a starch substrate and determining the activity of the α-amylase enzyme of the sample in the breakdown of the starch substrate. As used herein, the term "substrate tube" means a tube that contains a labeled starch substrate. In some embodiments, the sample is a flour sample. In some embodiments, the sample is a stock sample. In some embodiments, the sample is an amylase concentrate sample. In some aspects of the

invention, the starch substrate is detectably labeled. This detectable label is attached to the starch substrate utilizing standard chemistry methods and allows quantification of the amount of cleavage of the starch substrate by α-amylase after it is contacted with the sample. Such standard methods may include, but are not limited to, attaching a detectable label to the starch substrate through chemical conjugation. Various conjugation reagents including, but not limited to, cyanogen bromide activation or pryidinium dichromate oxidation, followed by reductive amination. In the case of cyanogen bromide activation, the activated starch will react with the amino groups of fluorescent materials to form the fluorescence-labeled substrate starch.

In some embodiments, the activity of the α-amylase is determined by quantifying the amount of detectably labeled starch substrate fragments that have been cleaved from the starch substrate. In other embodiments, the activity of the α-amylase is determined by quantifying the amount of detectably labeled starch substrate that remains intact following contact with the sample.

The invention involves in some aspects separating the soluble detectably labeled starch fragments from the detectably labeled starch in the reaction mixture. Methods that may be used to separate the fragments from the reaction mixture include, but are not limited to: filtration, centrifugation, and affinity binding methods. As used herein, the term "filtering" means passing the sample through one or more filter devices. Such devices include, but are not limited to paper filters, screens, mesh, etc. Filtering may involve passing the material to be filtered though a single filter, or through a multiple filters, which may be of the same type or may be of differing types (e.g., a screen followed by a paper or a mesh followed by a screen and/or paper filter). Filtration is done using standard methods known in the art. One of ordinary skill in the art will recognize there are numerous filtration methods, combinations, and techniques that are useful in the methods and kits of the invention. In the methods and kits of the invention, filtering methods may also include the use of filtration aids including, but not limited to: resins, beads including glass beads, and celite, which is also known as diatomaceous earth and Kieselguhr. Selection and use of such filtration aids in the methods of the invention, will be understood by one of ordinary skill in the art.

The invention relates in part to the use of centrifugation methods to separate detectably labeled fragments from the reaction mixture. Such methods include the centrifugation and may also include the removal of an aliquot of the supernatant of the centrifugation for measurement of the amount of detectably labeled starch substrate fragments. The removal of an aliquot from the centrifuged reaction mixture may be followed by the centrifugation of the aliquot prior to determination of the level of detectably labeled starch substrate in the aliquot.

The invention also relates in part to the use of affinity binding methods to separate detectably labeled fragments from the reaction mixture. An example of an affinity binding method, although not intended to be limiting, is the use of affinity chromatography methods to separate detectably labeled fragments from the reaction mixture. Affinity binding methods include the use of agents that bind to the molecules to be separated. Such agents include, but are not limited to, lectins and antibodies. As will be recognized by one of ordinary skill in the art, the agent may be bound to a support, e.g. as in affinity column chromatography. It will be understood that in alternative embodiments, the agent is not bound to a surface. Methods of separating molecules using methods such as affinity binding and/or affinity chromatography are well understood by those of ordinary skill in the art. Examples of affinity separation methods are provided in US Patent No. 6,362,008, which is hereby incorporated by reference in its entirety.

One of ordinary skill in the art will recognize that following a separation step as described herein, either the soluble detectably labeled substrate fragments, the detectably labeled substrate, or both, can be measured using the methods of the invention to determine the α-amylase activity in the sample tested. Such measurements may be done using standard methods, including, but not limited to, transferring the supernatant or filtrate samples to a measurement cuvette, followed by measurement on a calibrated fluorometer. In such readings, the fluorescent reading would be proportional to the amount of amylase presented in the flour, stock, or amylase concentrate samples.

As used herein the term "time sufficient for α-amylase to hydrolyze the starch substrate" means the amount of time for hydrolysis to occur. The time sufficient is at least about 1 sec, 5 sec, 10 sec, 15 sec, 20 sec, 25 sec, 30 sec, 35 sec, 40 sec, 45 sec,

50 sec, 55 sec, 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 11 min, 12 min, 13 min, 14 min, 15 min, 16 min, 17 min, 18 min, 19 min, 20 min, 21 min, 22 min, 23 min, 24 min, 25 min, 26 min, 27 min, 28 min, 29 min, 30 min, 31 min, 32 min, 33 min, 34 min, 35 min, 36 min, 37 min, 38 min, 39 min, 40 min, 41 min, 42 min, 43 min, 44 min, 45 min, 46 min, 47 min, 48 min, 49 min, 50 min, 51 min, 52 min, 53 min, 54 min, 55 min, 56 min, 57 min, 58 min, 59 min, or 60 min. Preferably, the time is at least 1 minute, at least 5 minutes, at least 10 minutes, or at least 15 minutes.

As used herein the term: "hydrolysis" means at least partial hydrolysis of the starch substrate. Total hydrolysis of the starch substrate is not required. As used herein, the term "soluble detectably labeled starch fragments" means fragments of the detectably labeled starch that have been released from the starch by hydrolysis. The soluble detectably labeled starch fragments are no longer attached to the starch substrate.

In some embodiments of the invention, a control sample, or amylase standard may be prepared. As used herein the terms "amylase standard" and "control sample" means a sample with a known amount of α-amylase activity that may be contacted with a starch substrate identical to that contacted with the flour or stock test sample. The reaction with the known amount of α-amylase activity thereby serves as a control reaction (or standard reaction) from which one of ordinary skill can extrapolate the level of activity in the test sample. One of ordinary skill in the art will recognize how to prepare and utilize a control or standard reaction to allow determination of the α-amylase activity in test samples.

The invention also includes in some aspects, the use of an α-amylase standard curve, which may include, for example, fluorescent values that correspond to a range of α-amylase concentrations. An α-amylase standard curve may be used to compare the value in a sample as a determination of the amount of α-amylase activity in the sample. In some embodiments, a "control" or "amylase standard" value is a value from an α-amylase standard curve.

The invention includes a starch substrate that is detectably labeled. As used herein, a "starch substrate" is a starch molecule upon which α-amylase acts enzymatically. As used herein, the term "starch" includes, but is not limited to, wheat

starch, waxy wheat starch, corn starch, waxy maize starch, oat starch, rice starch, tapioca starch, mung-bean starch, potato or high amylose starches, and sorghum starch. In preferred embodiments, the starch substrate is potato starch.. In some preferred embodiments, the potato starch is cross-linked potato starch.

As used herein, a starch substrate or starch substrate fragment that is "detectably labeled" means a starch substrate or substrate fragment to which a label that can be detected is attached. The term "label" as used here means a molecule preferably selected from, but not limited to, the group consisting of fluorescent, enzyme, radioactive, metallic, biotin, chemiluminescent, and bioluminescent molecules. As used herein, the label is not a colorimetric label, e.g., a chromophore molecule. In some aspects of the invention, a label may be a combination of the foregoing molecule types.

Radioactive or isotopic labels include, for example, ^{14}C, ^{3}H, ^{35}S, ^{125}I, and ^{32}P. Fluorescent labels include any compound that emits an electromagnetic radiation, preferably visible light, resulting from the absorption of incident radiation and persisting as long as the stimulating radiation is continued. Such compounds include coumarin containing molecules, and further include anthroyl compounds, naphthalene compounds, pyrene compounds, compounds containing benzyl, pyrenyl and phenyl groups, fluorescein compounds, anthracene compounds, compounds containing conjugated pi electron systems, but are not limited to these categories of compounds and include any compound that could be used as a label in this invention.

Examples of the fluorescent coumarin molecules include 7-hydroxycoumarin, 7-aminocoumarin, and further include 6-((7-amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid, succinimidyl ester, 7-amino-3-((((succinimidyl)oxy)carbonyl)methyl)-4-methylcoumarin-6-sulfonic acid, 7-diethylaminocoumarin-3-carboxylic acid, 7-diethylaminocoumarin-3-carboxylic acid succinimidyl ester, 7-diethylamino-3-(4'-isothiocyanophenyl)-4-methylcoumarin, 7-dimethylaminocoumarin-4-acetic acid, 7-dimethylaminocoumarin-4-acetic acid succinimidyl ester, 7-hydroxycoumarin-3-carboxylic acid, 7-hydroxycoumarin-3-carboxylic acid succinimidyl ester, 7-hydroxy-4-methylcoumarin-3-acetic acid, 7-hydroxy-4-methylcoumarin-3-acetic acid succinimidyl ester, 7-methoxycoumarin-3-

carboxylic acid, 7-methoxycoumarin-3-carboxylic acid succinimidyl ester, 7-diethylaminocoumarin-3-carbonyl azide and 7-methoxycoumarin-3-carbonyl azide.

Examples of naphthalene compounds include 5((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS), 6-((5-dimethylaminonaphthalene-l-sulfonyl)amino)hexanoic acid, 2-dimethylaminonaphthalene-5-sulfonyl chloride, dimethylaminonaphthalene-6-sulfonyl chloride, 6-(N-methylanilino)naphthalene-2-sulfonyl chloride, 6-(p-toluidinyl)naphthalene-2-sulfonyl chloride and 5-acenaphthalene.

Examples of other fluorescent labels include but not limited to 2,4-dinitrophenyl, acridine, cascade blue, rhodamine, 4-benzoylphenyl, 7-nitrobenz-2-oxa-1,3-diazole, 4,4-difluoro-4-bora-3a,4a-diaza-3-indacene and fluorescamine. Absorbance-based labels include molecules that are detectable by the level of absorption of various electromagnetic radiation. Such molecules include, for example, the fluorescent labels indicated above.

Chemiluminescent labels in this invention refer to compounds that emit light as a result of a non-enzymatic chemical reaction.

As used herein, fluorophores include, but are not limited to amine-reactive fluorophores that cover the entire visible and near-infrared spectrum. Examples of such fluorophores include, but are not limited to, 4-methylumbelliferyl phosphate, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), BODIPY dyes; Oregon Green, rhodamine green dyes; the red-fluorescent Rhodamine Red-X, Texas Red dyes; and the UV light–excitable Cascade Blue, Cascade Yellow, Marina Blue, Pacific Blue and AMCA-X fluorophores. Fluorophores may also include non-fluorescent dyes used in fluorescence resonance energy transfer (FRET).

In addition to alkaline phosphatase and peroxidase, other enzymes that can be used in methods and kits of the invention include, but are not limited to β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, galactose oxidase, glucose oxidase and hexokinase.

The labeled molecules of the invention can be prepared from standard moieties known in the art. As is recognized by one of ordinary skill in the art, the labeling process will vary according to the molecular structure of the detectable label. For fluorescent materials with free amino groups, a typical process, though not

intended to be limiting, would be to use alkaline cyanogen bromide to activate starch substrate. The cyanogen-bromide-activated starch reacts with the free amino group of the fluorophores to form a new bond, e.g. an isourea, carbamate, or imidocarbamate bond, which links the fluorophores onto the starch substrate. Other methods of labeling molecules with one or more of the above-identified types of detectable labels are routinely used and are well understood by those of ordinary skill in the art.

The invention involves, in some embodiments, a labeled starch substrate that is labeled on about one of every 300-1300 D-glucose residues of the starch. In certain embodiments, the starch substrate is labeled on about one of every 300-500, about one of every 500-700, about one of every 700-900, about one of every 900-1100, about one of every 1100-1300, or various combinations thereof. Smaller ranges also are contemplated, such as every 100 units (300-400, 400-500, etc.), every 50 units (300-350, 350-400, etc.), and so on.

The invention in another embodiment, includes measuring α-amylase activity in a flour, stock, or amylase concentrate sample by forming a reaction mixture by contacting the sample with a detectably labeled starch substrate attached to a surface, for a time sufficient for the α-amylase enzyme to hydrolyze the starch substrate. As used herein the term "surface" means a material including any synthetic or natural material. Examples of surfaces of the invention include, but are not limited to: glass, plastic, nylon, metal, paper, cardboard, and can be in numerous forms including, but not limited to, tubes, centrifuge tubes, cuvettes, cards, slides, dipsticks, beads, coverslips, multiwell plates, Petri plates, etc. One of ordinary skill in the art will recognize that numerous additional types of surfaces can be used in the methods of the invention.

As used herein the term "attached to a surface" means chemically or biologically linked to the surface and not freely removable from a surface. Examples of attachment, though not intended to be limiting are covalent binding between the surface and the starch substrate, attachment via specific biological binding, or the like. For example, "attached" in this context includes chemical linkages, chemical/biological linkages, etc. As used herein the term "covalently attached" means attached via one or more covalent bonds. As used herein the term "specifically attached" means a species is chemically or biochemically linked to a surface as

described above with respect to the definition of "attached," but excluding all non-specific binding. In the methods of the invention, a starch substrate that is attached to a surface is attached such that the substrate is not removable from the surface without specific stripping methods or solutions. Such stripping methods may include, but are not limited to, physical methods such as scraping or heating, enzymatic methods, and chemical methods, which may include but are not limited to contacting the attached substrate and surface with a solution such that the link between the substrate and the surface is broken and the substrate is released.

One of ordinary skill in the art will be able to envision the steps of forming a reaction mixture by contacting a detectably labeled starch substrate attached to a surface with an α-amylase enzyme and removing the labeled fragments from the reaction mixture. The amount of label present on the fragments released by the hydrolysis (soluble fragments) is measured and/or the amount of label that remains on the starch that has not been hydrolyzed and therefore remains attached to the surface is measured, and either or both measurements are to be compared to the initial amount of label on the surface prior to contact with the α-amylase enzyme. From a comparison of the levels of labeled starch before and after hydrolysis, a determination of the amount of α-amylase activity in the reaction mixture can be made. One of ordinary skill in the art will recognize that the total amount of detectably labeled starch prior to contact with the α-amylase, can be compared with either the level of label on pieces released by α-amylase hydrolysis, or the amount of detectably labeled substrate that remains attached to the surface following hydrolysis. This type of method can be used to determine the amount of α-amylase enzyme activity in the flour or stock sample or control sample.

The following illustrates the use of a method of the invention to determine the level of α-amylase activity in a flour, stock, and/or amylase concentrate sample. For example, if detectably labeled starch is contacted with a flour, stock and/or amylase concentrate sample for a time sufficient to hydrolyze the starch and subsequent measurement of the amount of detectably labeled starch substrate fragments that are not attached to the surface is determined to be zero, it indicates the absence of α-amylase activity in the flour, stock, and/or amylase concentrate sample tested. In addition, the determination that the original amount of detectably labeled starch

substrate that was attached to the surface remains attached to the surface, indicates that there is no α-amylase activity in the sample. In contrast, if the sample contains α-amylase, the enzyme will break down the starch substrate and the hydrolyzed substrate fragments will be released or solubilized. After separation of the hydrolyzed, small-sized starch fragments from the non-hydrolyzed starch substrate, fluorescence from either the starch fragments or the non-hydrolyzed starch substrate can be measured to determine quantity of α-amylase activity in the sample. The amount of α-amylase activity in the flour, stock, and/or amylase concentrate sample will be positively proportional to the fluorescent reading in the starch fragments, but inversely proportional to the fluorescence in the non-hydrolyzed starch substrate.

In some embodiments of the invention, the reaction mixture includes detectably labeled starch substrate attached to a surface such as a test tube or centrifuge tube, which is contacted with α-amylase in a sample. Following contact for a time sufficient for α-amylase to hydrolyze the starch substrate, the hydrolyzed starch substrate fragments that are not attached to the surface can be separated from the non-hydrolzyed substrate and measured, and/or the detectably labeled starch substrate that remains attached to the surface may be measured as attached to the tube, or may be stripped off the surface and its quantity determined. For example, to strip off the detectably labeled starch substrate attached to the surface, the surface may be treated with physical or chemical methods. The amount of stripped detectably labeled starch substrate is then collected and the level of labeled starch substrate is measured as a determination of the activity level of α-amylase in the sample. One of ordinary skill in the art will recognize that prior to determination the activity level, a purification step such as, but not limited to, centrifugation, filtration, or affinity binding methods, may be used to further separate the soluble detectably labeled fragments. Following the separation, a determination of the amount of soluble detectably labeled fragments and/or retained substrate is done. This determination may be done using a routine detection method, which can be selected based on the type of detectable label utilized. Examples of such methods, include, but are not limited to the use of a fluorometer to determine the amount of detectably labeled fragments or retained substrate when the label is fluorescence, or the use of a scintillation counter if the label is radioactive.

One of ordinary skill in the art will be familiar with the variety of detection systems that can be utilized in the methods of the invention.

The invention also relates in some aspects to kits for measuring α-amylase activity in a flour. stock, and/or amylase concentrate sample. The kits of the invention may include a first container of detectably labeled starch substrate, a second container of an α-amylase standard and instructions for measuring the α-amylase activity in a flour, stock, and/or amylase concentrate sample. Some kits of the invention may include a container containing a starch substrate, a second container containing an α-amylase standard, a third container containing a detectable label, instructions for labeling the starch substrate, and instructions for measuring the α-amylase activity in a flour, stock, and/or amylase concentrate sample. The kits of the invention may also include additional components such as tubes, vials, containers, dip sticks, buffers, water, fluorometer calibration standards, an α-amylase standard curve, etc. The kits of the invention may also be provided in conjunction with supplementary equipment (e.g. measuring devices such as fluorometers), and may also include instructions for running the assays of the invention utilizing the supplementary equipment.

Examples

Example 1

Introduction

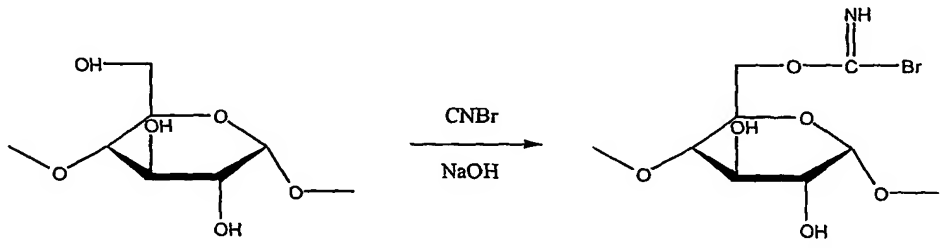
Wheat or fungal α-amylase activity in flour samples is tested. The method may also be used to measure the activity of other types of microbial α-amylase, such as bacterial α-amylase activity.

Methods

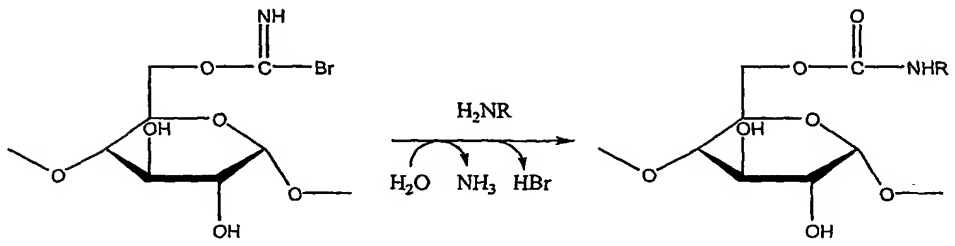
Preparation of a Fluorescent Starch Substrate for use in Assaying α-amylases

Starch, for example potato starch or waxy maize starch, is activated by reaction with alkaline cyanogen bromide using the method of Cuatrecasas, P. and Anfinsen, C., *Meth. Enzymol.* 22:351-378, 1971. Activation is followed by reaction with a fluorescent dye that has a free amino group. The amino group of the

fluorescent molecule reacts with the cyanogen-bromide-activated starch according to the following reactions, wherein R= ligand (e.g. fluorescent or enzyme).



polysaccharide activated polysaccharide



activated polysaccharide ligand or enzyme coupled to polysaccharaide

The amount of derivatization of the starch is kept to 1 in 300 to 1 in 1300 D-glucose residues to allow α-amylase to react with the derivatized starch. The labeling ratio can be determined by NMR using standard methods. It can also be determined by measuring the quantity of fluorescence labeling of D-glucose residues. The number of fluorescent molecules can be calculated by the absorbance coefficient, while the number of micromoles of glucose can be determined by the micro phenol-sulfuric acid method using standard procedures.

Assay for α-amylase Activity (A)

Water is added to a flour sample to liquefy and make a slurry. A known amount of prepared liquefied flour is added to an aliquot of prepared substrate (fluorescent starch). The mixture is incubated at 45°C. The reaction mixture is added to a filtration device that will retain the starch and flour particles but permit

hydrolyzed detectably labeled fragments to pass. The filtrate is mixed well and read in a calibrated fluorometer to determine amylase activity.

Assay for α-amylase activity (B)

1) 200 mg fluorescent starch is suspended in 1 ml buffer.
2) The 1-ml sample to be assayed is added to the starch.
3) The samples are mixed and reaction allowed to proceed.
4) The reaction mixture is then mixed again and centrifuged and aliquots are taken from the supernatant for measurement of the fluorescence. The level of fluorescence is proportional to the amount of α-amylase activity in the sample.
5) Alternatively, samples are continuously stirred and aliquots are taken as described in step 3, centrifuged, and fluorescence measured as in step 4.

Example 2

Methods

The following buffers were used as indicated in the Examples below.

Reaction Buffer Stock was prepared by dissolving 17.6ml Acetic acid, 16.0gm anhydrous sodium acetate, 29.2gm sodium chloride, and 5.6gm calcium chloride in de-ionized (DI) water to a final volume of 1 liter. The Reaction Buffer used in the assays was prepared by diluting the Reaction Buffer Stock 1:10 v/v with DI.

Stop Buffer was prepared by mixing 115.5ml acetic acid with DI water to a final volume of 1 liter.

Phosphate Buffer (Reaction Buffer for Cereal Amylase) was prepared by dissolving 1.2651gm anhydrous monobasic sodium phosphate and 1.562gm anhydrous dibasic sodium phosphate in DI to a final volume of 1 liter.

Substrate Tubes used in the reactions were AMYLease™ substrate tubes (Vicam, Watertown, MA) containing labeled starch substrate.

Test for Cereal Amylase

Introduction

The objective of this study was to measure cereal amylase activity.

The fluorometer was calibrated and 6ml Phosphate Buffer was added to each AMYLease™ substrate tube (Vicam). The substrate tubes were pre-warmed to 45°C. 200 mg of sample was weighed and added to the pre-warmed substrate tube. The mixture was incubated exactly 10 minutes at 45°C. 4 ml Stop Buffer was added to the tube following the incubation.

The incubation mixture was filtered through a microfiber filter into a clean cuvette. The fluorescence was read in the calibrated fluorometer and the fluorescence resulting values, which are shown in Table 1 were plotted versus the Falling Number values for a series of known samples (see Fig. 1).

Table 1. Cereal Amylase test results.

Falling Number	Fluorescence (ppm)
570	33
524	31
354	53
280	63
272	54
132	170
118	220
111	260

Example 3

Test For Fungal Amylase in Wheat Flour and Fungal Concentrates

Introduction

The objective of this study was to utilize to measure fungal amylase activity in wheat flour and in concentrated fungal amylase preparations (e.g., amylase concentrates). Tests were conducted with two types of commercial fungal amylase preparations. Doh Tone™ (American Ingredients, Inc. Anaheim, CA) contains 5.5% by weight neat fungal amylase, and it also contains fungal proteinases, wheat starch and silica. Doh Tone™ is usually dosed at 2g per 100lb wheat flour. Doh Tone™ II (American Ingredients, Inc.) contains 2.75% by weight of neat fungal amylase, wheat

starch and silica. Doh Tone™ II does not contain proteinases. Doh Tone™ II is usually dosed at 4g per 100lb wheat flour.

Example 3A

This study encompassed linearity, precision, and day-to-day repeatability for measurement of fungal amylase in wheat flour. Linearity was determined using results obtained from flour samples spiked with Doh Tone™ II at levels from 0.002% to 0.15 % by weight. Precision was determined by making triplicate measurements on flour samples spiked at 0.008% by weight with Doh Tone™ II and samples spiked at .004% by weight with Doh Tone™. Day-to-day repeatability over 4 days was assessed by testing flour samples spiked with Doh Tone™ II at 0.008%, 0.02%, 0.04%, 0.08% and 0.15% by weight.

Methods

Fungal Amylase in Wheat Flour

1. A 1mg/ml suspension of Doh Tone™ or Doh Tone™ II in Reaction Buffer was prepared.

2. A 3g (± .05g) flour sample to be tested was added to a 50-ml tube. The appropriate volume of Doh Tone™ suspension was added to give the desired final % by weight in the 3g flour sample. Reaction Buffer was added until the total weight of sample plus buffer was 30g (± .05g). The mixture was mixed well by capping and inverting the tube several times. The extract was then allowed to settle for 3-5 minutes.

3. 8 ml of the extract was placed in an empty tube and warmed to 45°C in a heating block. The heating took about 3-5 minutes.

4. The pre-warmed extract was added to a Substrate Tube. The Substrate Tube was capped and rapidly mixed by inverting tube 4-6 times, and placed in the heating block for a 10-min incubation.

5. After incubating exactly 10 minutes, 2ml Stop Buffer was added to the Substrate Tube. The Substrate Tube was capped and rapidly mixed by inverting tube 4-6 times.

6. For each sample, one microfiber filter (Vicam part # 31955, Vicam, Watertown, MA) was placed into a filter funnel (Vicam part # 36020). The incubation mixture was filtered through microfiber filters into a clean cuvette.

7. The fluorescence of samples was measured in a fluorometer calibrated using calibration standards (Vicam part# 33060) and with the high (red) standard set to 1000 ppm and the low (green) standard set to 0 ppm.

Results

Linearity

Linearity was determined using spiked wheat flour samples ranging from 0.002% to 0.15 % Doh Tone™ II by weight run as described above. Table 2 illustrates the results at different Doh Tone™ percentages in flour. Fig. 2 is a graph that of the linear regression analysis equation for the fluorescence reading versus the amount of Doh Tone™ II spiked. The correlation coefficient (r) of 0.998 from the above linear regression equation indicates that the linearity of this method is very good for Doh Tone™ II in flour in the range 0.002% to 0.15% by weight.

Table 2. Results of tests to determine linearity of assays

Doh Tone™ Percentage in Flour (Karl98)	Fluorescence-ppm
0	110
0.002	120
0.004	130
0.008	150
0.02	200
0.04	280
0.08	490
0.1	580
0.15	850

Precision

Precision was determined using the method provided above. Triplicate measurements were made on flour samples spiked at 0.008% by weight with two different lots of Doh Tone™ II and on samples spiked at .004% by weight with two different lots of Doh Tone™. Table 3 shows the results of this study. The results above show a good precision for different lots of commercial fungal amylase preparations in flour or neat in buffer.

Table 3. Results of tests to determine assay precision

Samples	Lot#	Repeat-1	Repeat-2	Repeat-3	Average	STDEV	C.V (%)
Flour K99 w/o Doh Tone™		95	90	89	91.33	3.21	3.52%
Flour K99 w/ 0.004% Doh Tone™	2253	130	120	120	123.33	5.77	4.68%
Flour K99 w/ 0.004% Doh Tone™	3035	140	130	130	133.33	5.77	4.33%
Flour K99 w/ 0.008% Doh Tone™ II	2225	130	120	120	123.33	5.77	4.68%
Flour K99 w/ 0.008% Doh Tone™ II	3035	140	130	130	133.33	5.77	4.33%

w/o = without; w/ = with; STDEV = standard deviation; C.V. = coefficient of variance

Repeatibility

Day-to-day repeatability over 4 days was assessed by testing flour samples spiked with Doh Tone 2 at 0.008%, 0.02%, 0.04%, 0.08% and 0.15% by weight using the method provided above. Table 4 shows the results of this study. The results indicate a good repeatability of measurements of commercial fungal amylase in flour samples.

Table 4. Results of tests to determine repeatability of assays.

Samples	FL. Day-1	FL Day-2	FL Day-3	FL Day-4	Average	STDEV	C.V. (%)
Flour K98 w/o Doh Tone™ (DT)	100	110	110	99	104.75	6.08	5.80%
Flour K98 w/ 0.008% DT C416	140	150	140	140	142.50	5.00	3.51%
Flour K98 w/ 0.02% DT C416	190	200	190	190	192.50	5.00	2.60%
Flour K98 w/ 0.04% DT C416	280	280	280	280	280.00	0.00	0.00%
Flour K98 w/ 0.08% DT C416	490	490	500	500	495.00	5.77	1.17%
Flour K98 w/ 0.15% DT C416	n/a	850	930	890	890.00	40.00	4.49%

w/o = without; w/ = with; FL = Fluorescence; STDEV = Standard deviation; C.V. = coefficient of variance

Example 3B

This study determined linearity and precision of measurements of concentrated commercial preparations of fungal amylase. Linearity was determined using results obtained from diluted suspensions of Doh Tone™ II over a concentration range of 0.01 to 1.0 gm/ml. Precision was assessed using triplicate measurements of 0.1 mg/ml solutions of Doh Tone™ I and Doh Tone™ II.

Concentrated Fungal Amylase Procedure

1. Dilution steps were required for commercial preparations that may contain 2-5% by weight of neat fungal amylase.

2. The fluorometer was calibrated suing calibration standards (Vicam part# 33060) and with the high (red) standard set to 1000 ppm and the low (green) standard set to 0 ppm.

3. 200 mg (±1mg) of fungal amylase preparation to be tested was placed in a 50-ml tube. 20ml Reaction Buffer was added to the tube. The tube was capped and

mixed well by inverting the tube several times. 2ml of the this first dilution was added to another tube and diluted with 18 ml Reaction Buffer.

4. 1.6 ml of the second dilution was placed in an empty tube, 6.4 ml Fungal Incubation Buffer was added, and the tube was warmed to 45°C in the heating block. The warming took from 3-5 minutes.

[*Note*: In some experiments, a series of further dilutions of the second dilution and Reaction Buffer were prepared to create a range of concentrations to obtain a concentration within range of about 0.1-1.0 Sandstedt-Kneen-Blish (SKB) unit/ml for testing (see Figs. 2-4). An SKB unit is an α-amylase dextrinizing unit (DU), and is defined as the quantity of α-amylase that will dextrinize soluble starch in the presence of an excess of β-amylase at the rate of 1 gram per hour at 30°C [see Institute of Medicine Food Chemicals Codex, 4th Ed., pp. 1451-1454, National Academy Press, Chapman & Hall, CRC netBASE; and R.M. Sandstedt, et al., Cereal Chemistry 16:712-723 (1939)]. The steps provided below were followed for each of the dilutions to determine the amount of amylase at each dilution].

5. The prewarmed extract was added to a Substrate Tube, which was capped and rapidly mixed by inverting tube 4-6 times. The Substrate Tube was placed in the heating block for a 10-minute incubation.

6. After incubating exactly 10 minutes, 2ml of Stop Buffer was added. The Substrate Tube was capped and rapidly mixed by inverting tube 4-6 times.

7. For each sample, one microfiber filter (Vicam part # 31955) was placed into a filter funnel (Vicam part # 36020). The incubation mixture was filtered through microfiber filters into a clean cuvette.

8. The fluorescence of samples was measured in a fluorometer calibrated using calibration standards (Vicam part# 33060) with the high (red) standard set to 1000 ppm and the low (green) standard set to 0 ppm.

9. In some instances, the fluorescence value of a sample is converted into an Enzyme Units Equivalent value. The conversion is done by reading off a plot (e.g. standard curve) of fluorescence value versus fungal amylase concentrations standardized in SKB units, which is generated using a series of fungal amylase samples of known concentrations. The standard curve is used to determine the equivalent Enzyme Unit value for a test (unknown) sample, based on the fluorescence determined for the sample using the α-amylase assay.

The precision and linearity of measurement of concentrated fungal amylase preparations was determined using the procedure described above.

Linearity of measurements of concentrated fungal amylase

Linearity was determined using results obtained from diluted suspensions of Doh Tone™ II over a concentration range of .01 to 1 mg/ml using the procedure described above (See Table 5). Fig. 3 shows the determination of Pure Doh Tone™ II with the assay. As expected for a substrate-based enzyme assay there is a working range, which appears to be from 0.01 to 0.1 mg/ml of Doh Tone™ II. Fig.4 and Fig. 5 show the curves of the quadratic and linear relationship of fluorescence to Doh Tone™ concentration in mg/ml), respectively. Within the working range, the curve is better fit by a quadratic than by a linear relationship.

Table 5. Results of linearity tests

Doh Tone™ (DT) Conc. (mg/ml)	Fluorescence (ppm)
0	14
0.01	17
0.02	22
0.04	31
0.05	38
0.08	60
0.1	78
0.15	150
0.2	230
0.3	410
0.4	600
0.5	770
0.6	930

Precision of measurements of concentrated fungal amylase

Triplicate measurements were made of Doh Tone™ II or Doh Tone™ at concentrations of 0.1 mg/ml. Table 6 shows the results of this study. The results indicated a good precision for different lots of commercial fungal amylase preparations in flour or neat in buffer.

Table 6. Results of tests to determine assay precision using fungal concentrate

Samples	Lot#	Repeat-1	Repeat-2	Repeat-3	Average	STDEV	C.V (%)
0.1mg/mL Pure Doh Tone™	3097	330	330	340	333.33	5.77	1.73%
0.1mg/mL Pure Doh Tone™ II	2213	100	110	100	103.33	5.77	5.59%

STDEV = standard deviation; C.V. = coefficient of variance

Discussion Examples 3A and 3B

Linearity

The linearity using this method for fungal amylase in wheat flour is very good as indicated by the correlation coefficient (r) of 0.998.

Precision

The results of the assays showed a very good precision for fungal amylase in flour with CVs less than 5% for Doh Tone™ II concentrations ranging from 0.008% to 0.15%

Repeatability

Repeatability for fungal amylase in flour was very good with coefficients of variance (C.V.s) ranging from 0.0% to 4.49% for different concentrations of amylase.

Linearity for Concentrated Fungal Preparation

The concentrated preparation showed an excellent fit to a quadratic relationship $R^2 = .999$ and a good fit ($R^2 = 0.98$) to a linear relationship.

Example 4

Comparisons of different lots of Doh Tone™ and Doh Tone™ II (American Ingredients, Inc) were done using the methods described above. Results from various lots are shown in Table 6.

Table 6. Results of assay comparison of Doh Tone™ and Doh Tone™ II

Doh Tone™ (0.2mg/ml)	Fluorescence (ppm)
DT LOT # 415303601	860
DT LOT # 415303501	830
DT LOT # 415309701	830
DT LOT # 415225301	630
DT-II LOT # 416221301	260
DT-II LOT # 416222501	260
DT-II LOT # 416303501	300
DT-II LOT # 416227401	230
DT-II LOT # Code 416	260
DT-II LOT # 416022101 (o)	320
DT LOT # 415022101 (o)	840

DT = Doh Tone™

Example 5

Tests were performed to compare results of the amylase assay performed on flour supernatant versus flour suspension. Various flour samples with different percentages of Doh Tone™ and Doh Tone™ II were tested using the methods provided above (see Example 3). In that procedure the sample was allowed to settle during the extraction procedure and an 8ml sample of the supernatant was taken for pre-warming. Duplicate samples were tested using a modification of the extraction method whereby the sample was rapidly mixed by inverting the tube 4-6 times prior to taking an 8ml sample for pre-warming. The results are provided in Table 7 and a graph of the comparison of the results is shown in Fig. 6. The results indicate that

there is a consistent proportional increase in the fluorescence if a whole suspension of extract is used as compared to a supernatant.

Table 7. Results of sample supernatant versus sample suspension assay.

Samples	Suspension	Supernatant	Difference
Flour Karl 99	130	100	30
0.0005% DT in K99	140	110	30
0.008% DT II in K99	160	130	30
0.04% DT II in K99	270	230	40
0.08% DT II in K99	500	400	100
0.001% DT in K99	160	130	30
0.0005% DT in K99	140	110	30

K99 = flour sample Karl 99; DT = Doh Tone™

Example 6

A stability test was performed on an amylase substrate (Vicam, lot 03PF15006) at elevated temperature using the assay methods provided above. Flour samples (K98) were dosed with various amounts of fungal amylase (FA). The samples were then assayed as described in Example 3 above using substrate stored normally or substrate that had been kept at 37°C for 4 days as an accelerated stability condition. The results are shown in Figure 7.

Example 7

Tests were conducted using the methods described above to determine the working range of the amylase assay on fungal amylase from Sigma (St. Louis, MO). Results are shown in Fig. 8A and 8B, which show the results in fluorescence per unit of the Sigma fungal amylase. Fig. 9A and 9B are graphs of the results of the amylase assay on Sigma fungal amylase at high range of amylase units (Fig. 9A) and a low range of amylase units (Fig. 9B).

Example 8

Tests were run to determine the effects of native cereal amylase activity already in flour on the determination of fungal amylase using the amylase assay. Flours with different amounts of native cereal amylase activity (Falling Number from

250-524) were spiked with fungal amylase at various dosages and assayed using the amylase assay as described in Example 3. Most of the signal was contributed by the native cereal amylase. The signal from an untreated flour blank was subtracted from the total value to obtain a value for the added fungal amylase. The results are indicated in Fig. 10 and Table 8.

Table 8. Results of amylase assay of fungal amylase in flours containing different amounts of native cereal amylase activity

DT II Conc (%)	Buffer	FN 524	FN 354	FN 272	FN 250	Buffer (-B)	FN 524 (-B)	FN 354 (-B)	FN 272 (-B)	FN 250 (-B)
0	16	99	315	520	625	0	0	0	0	0
0.004	18	120	325	525	615	2	20	10	5	
0.008	21	140	350	550	670	5	40	35	30	45
0.015	25.5	170	375	590	690	9.5	70	60	70	65
0.02	29	195	400	630	785	13	95	85	110	160
0.04	44	290	560	720	925	28	190	245	200	300

DT II = Doh Tone™ II; FN = Falling Number value; (-B) = value minus the blank

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference in their entirety.

We claim: